Lethal factor of *Clostridium histolyticum* kills cells by apoptosis

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Abstract

In this study, for the first time, *Clostridium histolyticum* lethal factor was purified by ammonium sulfate precipitation of culture broth, centrifugation through an Amicon filter device and hydrophobic interaction chromatography. The purified lethal factor was devoid of proteolytic activity. At a concentration of 150 ng mL^{-1} the lethal factor killed 50% of HeLa cells within 24 h of exposure. Abrogation of actin filaments, activation of caspases, fragmentation of nuclear DNA as well as ultrastructural changes indicated that the cell death occurred by apoptosis. The apoptotic action of the lethal factor is in agreement with changes induced in animal tissues by administration of *C. histolyticum* culture medium.

Introduction

After World War II *Clostridium histolyticum* infections were rarely observed as a clinical problem. However, in recent years clinical reports concerning infections caused by this microorganism have included those on infective endocarditis (Durmaz *et al*., 2000), necrotic infections in drug users (Brazier *et al*., 2004) and necrotizing fasciitis (Thirumaran *et al*., 2005). Pathogenesis as a result of these infections is always connected with tissue lysis caused by toxic enzymes produced by *C. histolyticum* (Mandl *et al*., 1957; Kono, 1968; Mitchell & Harrington, 1968; Takahashi *et al*., 1970; Kessler & Yaron, 1973; Sparrow & McQuade, 1973; Bicsak & Harper, 1985; Mookhtiar & van Wart, 1992; Ullmann & Jakubke, 1994; Yoshihara *et al*., 1994; Martirosian *et al*., 2005). Culture broth filtrates of *C. histolyticum* contain collagenase (Mandl *et al*., 1957; Kono, 1968; Mookhtiar & van Wart, 1992), clostripain (Mitchell & Harrington, 1968; Ullmann & Jakubke, 1994), elastase (Takahashi *et al*., 1970), nonspecific peptidases (Mandl *et al*., 1957), proteinases (Sparrow & McQuade, 1973; Bicsak & Harper, 1985), aminopeptidase (Kessler & Yaron, 1973) and gelatinase (Yoshihara *et al*., 1994). Some strains of *C. histolyticum* also produce lethal and hemolytic factors of variable heat stability and susceptibility to oxidation (Steward, 1936; Bowen, 1952). The lethal activity of the supernatant disappears rapidly after the logarithmic growth phase probably owing to inactivation by proteolytic enzymes (Steward, 1936). After partial purification (Shemanova *et al*., 1967), one lethal factor was found to be toxic to cultures of chick embryo cells (Shamraeva *et al*., 1967). Furthermore, lethal factor was sometimes found to be present in commercial collagenase preparations killing, for example, beta cells during isolation of the islets of Langerhans (Moskalewski *et al*., 1973). Until now, however, purification of this factor has not been successful. While trying to purify this lethal factor we have managed to identify and describe a vacuolating toxin (Jankowska-Steifer *et al*., 2006). In the present paper we report on the purification of the lethal factor and propose a mechanism of action.

Materials and methods

Purification of lethal factor

The lethal factor was isolated from the supernatant of *C. histolyticum* (strain ATCC 19401) cultured in brain heart infusion medium (BHI; bioMérieux, Marcy L'Etoile, France) and lasted for 15–18 h. Longer cultivation results in complete loss of activity of the lethal factor (Steward, 1936; Bowen, 1952). Before centrifugation of the culture broth zinc chloride was added to a final concentration of 10 μM, in order to inhibit aminopeptidase (Kessler & Yaron, 1973). After ammonium sulfate precipitation (60 g 100 mL^{-1}) the sediment was dissolved in 15 mL distilled water. To the precipitate from 100 mL of culture broth supernatant 1 mg of *N*-tosyl-L-lysine-chloromethyl ketone (TLCK; Sigma-

Aldrich, St Louis, MO) was added in order to inhibit clostripain. Dissolved supernatant was then spun in an Amicon Ultra-15 centrifugal filter device with a 100 kDa nominal molecular weight limit (Millipore, Billerica, MA) at 4 °C. The filtrate was spun again in a filter device with a 30 kDa maximum weight limit. The retentate was reconstituted in 100 mM sodium phosphate with 2 M ammonium sulfate, centrifuged again and subjected to methyl hydrophobic interaction chromatography (methyl HIC; Bio-Rad, Hercules, CA) in 100 mM sodium phosphate with a gradient from 2 M to 0 M ammonium sulfate. Eluted fractions were desalted in 30-kDa filter device. Protein estimation was performed using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

Titration of toxin

Each batch of purified toxin was titrated against HeLa cells. The cells were seeded into 24 well plates (Corning Inc., Corning, NY) at a density at 5×10^4 cells well^{-1} in Dulbecco's modified Eagle medium culture medium supplemented with 10% fetal calf serum and antibiotics (penicillin G sodium, streptomycin sulfate, and amphotericin B in 0.85% saline; Gibco BRL, Paisley, UK). After overnight culture, the cells were exposed to increasing dilutions of toxin dissolved in sterile phosphate-buffered saline (Biomed, Lublin, Poland) mL^{-1} medium for 24 h. The cultures were then treated with 0.1% of crystal violet in 30% methanol for 10 min, rinsed with distilled water and dried. To estimate the binding of crystal violet the cells remaining in the wells were solubilized with 2% Triton X-100 (Sigma Aldrich Chemie, Diesenhofen, Germany) and the absorbance of solution measured at 540 nm in a Spectra II microplate photometer (SLT Labinstruments Deutschland GmbH, Crailsheim, Germany) (Flick & Gifford, 1984). The amount of toxin killing 50% of the cells (LD_{50}) was defined as a unit of activity.

Determination of protease activity

Protease activity in the supernatant of *C. histolyticum* cultures and purified lethal toxin was determined with the Protease fluorescence detection kit (Sigma) according to the method of Twining (Twining, 1984). Fluorescein isothiocyanate (FITC)-labeled casein served as the substrate. Fluorescence was determined in Fluoroskan Ascent FL (Thermo Labsystems, Helsinki, Finland) using an excitation wavelength of 490 nm and an emission wavelength of 525 nm. Enzymatic activity was expressed in fluorescent units μg^{-1} protein.

Estimation of clostripain activity

Clostripain enzymatic activity was estimated according to the method described by Porter (Porter *et al.*, 1971) before and after the addition of TLCK. Enzyme activity was measured as an increase in absorbance at 253 nm caused by the hydrolysis of *N*-benzoyl-L-arginine ethyl ester (BAEE; Sigma-Aldrich). It had been estimated that 1 U of clostripain hydrolyses one micromole of BAEE min^{-1} at room temperature and pH 7.6 (Fettucciari *et al.*, 2000). Prior to evaluation TLCK (final concentration 200 μM) was added to respective samples, and the enzyme was activated by a 3-h incubation at room temperature of 0.2–0.8 clostripain units per milliliter of 2.5 mM dithiothreitol (Sigma-Aldrich) dissolved in phosphate-buffered saline (PBS). One hundred microliters of this solution was added to 3 mL of 2.5 mM dithiothreitol and 0.25 mM BAEE in 0.075 M PBS (pH 7.6). Absorbance was measured for 5 min in 30-second intervals, at a wavelength of 253 nm in an Ultrospec 4000 UV/visible spectrophotometer (Pharmacia Biotech, Cambridge, UK). An absorbance curve was plotted and ΔA min^{-1} was determined from the linear part of the curve.

Polyacrylamide gel electrophoresis

Proteins in Tris/glycine running buffer (25 mM Tris, 192 mM glycine, pH 8.3 with or without 2-mercaptoethanol) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12% acrylamide) run at 90 V on a Mini Protean II apparatus (Bio-Rad) and stained with Coomassie blue. The Kaleidoscope Protein Standard (Bio-Rad) served as a reference.

Light microscopic observations and actin immunocytochemistry

HeLa cells grown on 12-mm coverslips were exposed to 1 or 2 U of lethal factor for 1, 2, 4 or 24 h. The cells were fixed in 2% paraformaldehyde dissolved in PBS for 10 min at room temperature, and rinsed three times in PBS. For phase-contrast observations the cells were mounted in Fluorescent Mounting Medium (Dako–Cytomation, Glostrup, Denmark). For actin detection the cells were permeabilized in methanol with 2 mM EDTA at − 20 °C, rinsed in PBS and treated with an antiactin mouse monoclonal antibody (Sigma) followed by $F(ab')_2$ fragmented rabbit antimouse immunoglobulin conjugated with fluorescein isothiocyanate (Dako-Cytomation). The cells were inspected using a Nikon Eclipse 800 fluorescence microscope and photographed with a Coolpix995 digital camera.

Transmission electron microscopy

The medium from dishes with control cells or exposed to 1 or 2 U of lethal factor for 3, 6 or 24 h was collected, centrifuged at 150 ***g*** to collect the detached cells and the cell pellet was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 30 min. Cells remaining in the dishes were

rinsed twice in PBS, and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 30 min. The cells were harvested with a rubber policeman, transferred into Eppendorf tubes and spun down. The cell pellets were left in fixative for a further 90 min. After fixation the cells were rinsed in cacodylate buffer, and postfixed in 1% osmium tetroxide for 2 h. The cells were then dehydrated in ethanol followed by propylene oxide and embedded in epoxy resin (PoliBed 812, Polysciences Europe GmbH, Eppelheim, Germany). Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined in a Jeol 100S electron microscope.

Determination of neutral red uptake

The cells were exposed to lethal factor at a concentration of 20–320 ng mL^{-1} for 1, 2 or 4 h. They were then incubated for 8 min at room temperature in PBS with 0.3% bovine serum albumin (BSA) and 0.05% neutral red (Sigma). The cells were rinsed three times in PBS with BSA and the neutral red was extracted with 70% ethanol and 0.37% HCl. Absorbance was read as the difference at 540 and 405 nm in a Spectra II microplate photometer (Jankowska-Steifer *et al.*, 2006).

The caspase activation assay

The caspase-mediated induction of apoptosis was determined at two consecutive stages. To assess the upstream caspase-9 activation in lethal factor-treated HeLa cells the Caspase-9 Staining Fluorescent Sulforhodamine BioAssay Kit (USBiological, Swampscott, MA) was used according to the protocol provided by the manufacturer. Briefly, cells were incubated with 1 U of lethal factor for 0.5, 1, 2, 4 and 6 h. The controls for the assay consisted of actinomycin D (1 μg mL^{-1})-treated cells (positive control) or cells cultured in medium alone (negative control). Then, cells were incubated with Red-LEHD-FMK substrate for 1 h followed by fluorescence-positive cell assessment at 510–560 nm using a Nikon Eclipse E800 (Nikon, Japan).

For the analysis of downstream caspase activation, the ApoFluor®Green Caspase Assay (Enzyme Systems Products, Livermore, CA) was used. Cells were cultured in the presence of 1 U of lethal factor for 6, 12 and 18 h followed by 1 h of incubation with the pan-caspase fluorescent substrate (FAM-VAD-FMK). The cells were then evaluated using a Nikon Eclipse E800 fluorescence microscope at the excitation wavelength and the percentage of apoptosis-positive fluorescent cells was determined.

DNA fragmentation assay (TUNEL)

DNA fragmentation in HeLa cells was analysed after 12, 18 and 24 h of incubation with 1 U of lethal factor using the APO-DIRECT^{TM} assay (Becton-Dickinson/PharMingen, San Diego, CA) according to Darżynkiewicz *et al.* (1997). Cells were washed twice with ice-cold PBS and fixed with 1% paraformaldehyde in PBS, followed by overnight incubation in ice-cold 70% ethanol. The cells were then stained with FITC-labeled deoxyuridine triphosphate (dUTP) using terminal deoxynucleotidyl transferase (TdT). The cells were washed twice and the nuclei costained with 4,6-diamidino-2-phenylindole (DAPI) using DAPI-containing Vectashield® Mounting Medium (Vector Laboratories, Burlingame, CA) and subsequently analysed using a Nikon Eclipse E800 fluorescence microscope. The percentage of apoptotic cells was calculated as a ratio of green (FITC-labeled) to blue (DAPI-labeled) fluorescent nuclei.

Results

Lethal factor characterization

Supernatant of *C. histolyticum* culture medium, either fresh or after addition of TLCK, displayed marked proteolytic activity. No activity could be detected in the purified lethal factor (Fig. 1a).

Samples of purified toxin (treated with TLCK) did not show any enzymatic activity towards BAEE. In this case absorbance of the reacting mixture remained at zero level during the whole period of spectrophotometric observation (Fig. 1b). Crude supernatant did hydrolyse BAEE and the kinetics of the reaction was more linear during the whole observation period (5 min). Addition of TLCK to crude supernatant caused a significant decrease of enzymatic activity. In addition, in this case absorbance changed in a linear fashion during the period of observation.

PAGE indicated that the purified lethal factor had an M_r ~of 63 kDa. It was not influenced by β–mercaptoethanol treatment of samples.

The toxic activity of the lethal factor studied by the crystal violet test was manifested at a concentration of 60 ng mL^{-1} and linearly increased with all cells killed at 320 ng mL^{-1} concentration (Fig. 2).

Morphological observation

Exposure to lethal factor caused contraction of cells and the appearance of apoptotic bodies. Cells showing such changes could be observed even after 4 h of exposure and the full effect was evident after 24 h (Fig. 3). Thick actin filaments formed irregular networks in control cells, which was particularly evident at the cell periphery. Actin was also present in thin spikes extending from the cell body. After 1 h of exposure, in numerous cells the actin network was partially disrupted and clouds of amorphous actin appeared in the cell centre. Cell extensions usually disappeared. Some cells became rounded and were completely devoid of

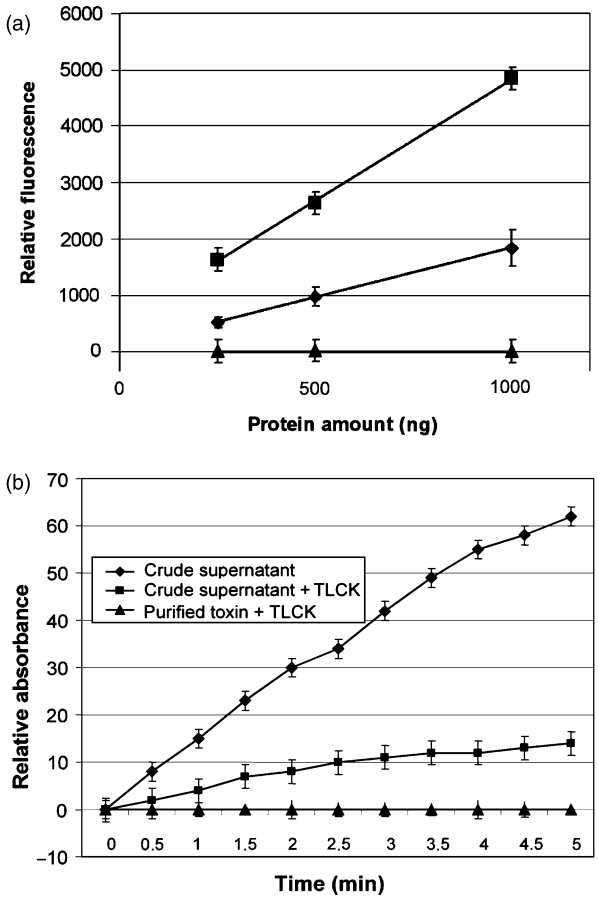

Fig. 1. Demonstration of enzymatic activity of *Clostridium histolyticum* culture supernatant. (a) General protease activity measured in samples of fresh *C. histolyticum* culture supernatant (■), supernatant with TLCK (◆) or purified toxin (▲). All samples were measured in three dilutions, containing 250, 500 or 1000 ng of protein. (b) Activity of clostripain measured as relative absorbance at 523 nm. Relative absorbance was calculated as the difference between absorbance after individual time intervals and basal absorbance at zero time. Error bars represent SD.

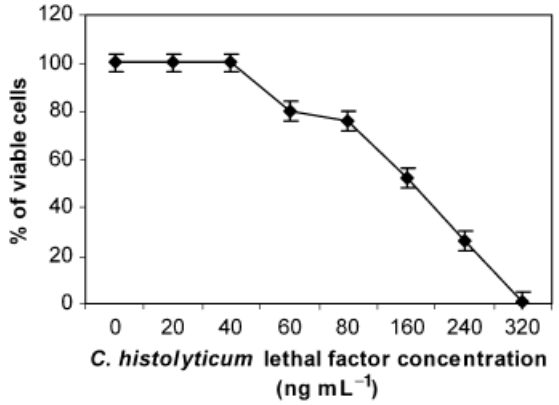

Fig. 2. Viability of HeLa cells exposed to *Clostridium histolyticum* lethal factor for 24 h, as determined by the crystal violet test. The unit of activity was defined as the amount of lethal factor killing c. 50% of cells (LD_{50}). It corresponded to 150 ng of toxin mL^{-1} culture medium.

filamentous actin. After 2 and 4 h exposure most cells had a completely fragmented actin network with actin filaments bent into loops (Fig. 4).

Under the electron microscope, a few apoptotic cells were seen after 3 or 6 h of exposure and were found to predominate after 24 h. Apoptotic cells displayed condensation of the chromatin and fragmentation of the nuclei with formation of micronuclei and apoptotic bodies. The density of cytoplasm increased considerably in comparison with control cells and intense blebbing was observed. Furthermore, in some cells large numbers of conspicuously dilated cisternae of smooth endoplasmic reticulum could be seen. Some of these cisternae remained elongated, whereas others assumed a circular shape. Elongated cisternae could have originated from the Golgi complexes, which were evident in control cells and disappeared in exposed cells. Furthermore, in some cells a number of vacuoles containing moderately electron-dense material or myelin-like structures were present. Rough endoplasmic reticulum and mitochondria appeared not to be affected. (Fig. 5). Exposure to the lethal factor (up to a concentration of 320 ng mL^{-1}) did not increase uptake of neutral red with respect to controls without the lethal factor (data not shown).

Caspase activation and DNA fragmentation assays

The assessment of caspase-9 activation revealed a time-dependent increase of red fluorescence intensity in lethal factor-treated HeLa cells. The observed fluorescence after 4 h of incubation of HeLa cells with 1 U of lethal factor was much brighter than that of an actinomycin D-treated (1 μg mL^{-1}) positive control (Fig. 6).

Involvement of the caspase cascade in the lethal factor-mediated induction of apoptosis in HeLa cells was further confirmed by an analysis of pan-caspase activation. It was found that 1 U of lethal factor induced maximal pan-caspase activation in 55% of cells after 18 h of incubation (Fig. 7a). Similarly, as shown by the TUNEL assay, HeLa cells exposed to 1 U of lethal factor for 18 h displayed extensive DNA fragmentation of *c.* 50% cells (Fig. 7b).

Discussion

Supernatant of *C. histolyticum* culture medium contains several proteolytic enzymes, such as clostripain (Mitchell & Harrington, 1968), elastase (Takahashi *et al.*, 1970), non-specific peptidases (Mandl *et al.*, 1957), proteinases (Sparrow & McQuade, 1973) and gelatinase (Yoshihara *et al.*, 1994). As all these enzymes are active against casein under test conditions employed (Twining, 1984), lack of

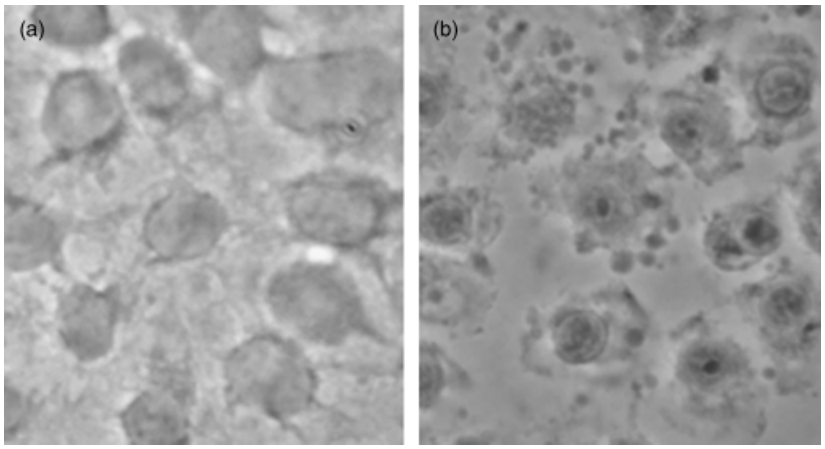

Fig. 3. Phase-contrast images of HeLa cells: (a) control and (b) exposed to 1 unit of lethal factor for 24 h. In (b) contraction of cells and numerous apoptotic bodies are evident. Magnification, × 400.

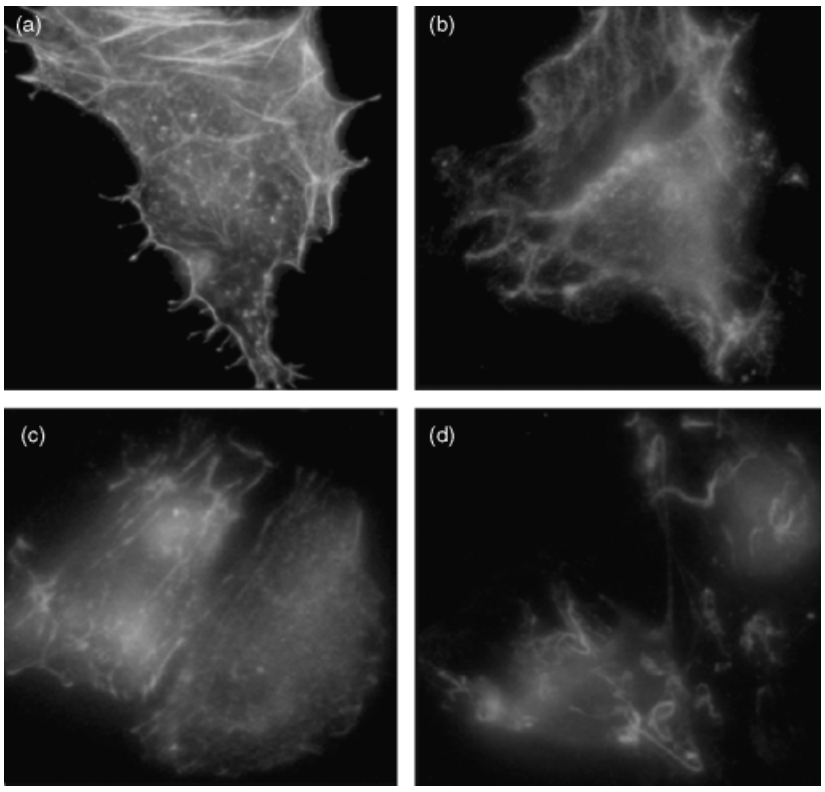

Fig. 4. Actin in HeLa cells exposed to lethal factor. A control cell (a) contains a network of distinct actin filaments whereas exposure to lethal factor for 1 (b), 2 (c) or 4 h (d) progressively disrupts the actin cytoskeleton.

proteolytic activity in the lethal factor samples allows us to exclude its contamination by these enzymes. Purification of the lethal factor required inhibition of clostripain (serine protease) with TLCK, but because this agent protected rather than inhibited the cytotoxic activity of *C. histolyticum* (Jóźwiak *et al.*, 2005, 2006), it does not seem likely that the lethal factor was affected by TLCK.

During the effector phase, apoptosis induces stimuli that activate a few typical pathways, resulting in the irreversible damage of cells (Kroemer *et al.*, 1995). Initiation of apoptosis may be accomplished by the extrinsic transduction pathway involving activation of a death receptor by a ligand leading to activation of initiator caspase-8 (Rokhlin *et al.*, 1998) or by the intrinsic pathway, which is a consequence of cellular stress leading to caspase-9 activation (Saleh *et al.*, 1999). Numerous bacterial toxins (Weinrauch & Zychlinsky, 1999; Gao & Abu Kwaik, 2000) among *Clostridium* spp., especially *Clostridium difficile* toxins A and B, are well recognized as agents triggering apoptosis (Fiorentini *et al.*, 1998; Brito *et al.*, 2002).

The effector phase of apoptosis involves activation of caspases representing a group of proteases that specifically

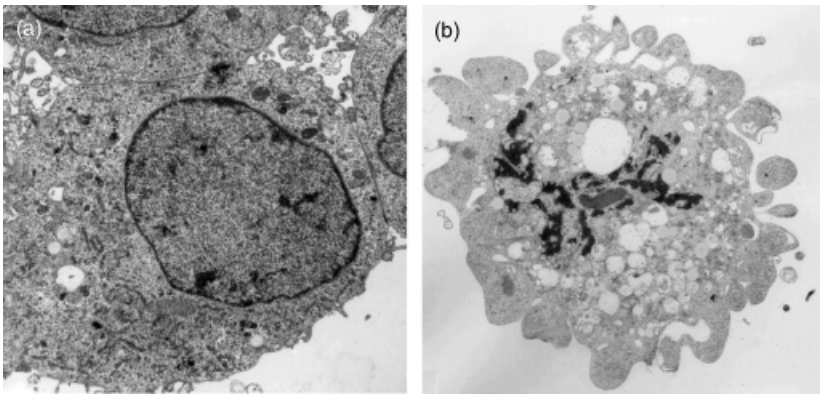

Fig. 5. HeLa cells: (a) control and (b) exposed to 2 U of lethal factor for 24 h. In (b) fragmentation of the nucleus, vacuolization of the cytoplasm, disappearance of microvilli and formation of numerous blebs is visible. Magnification, × 7100.

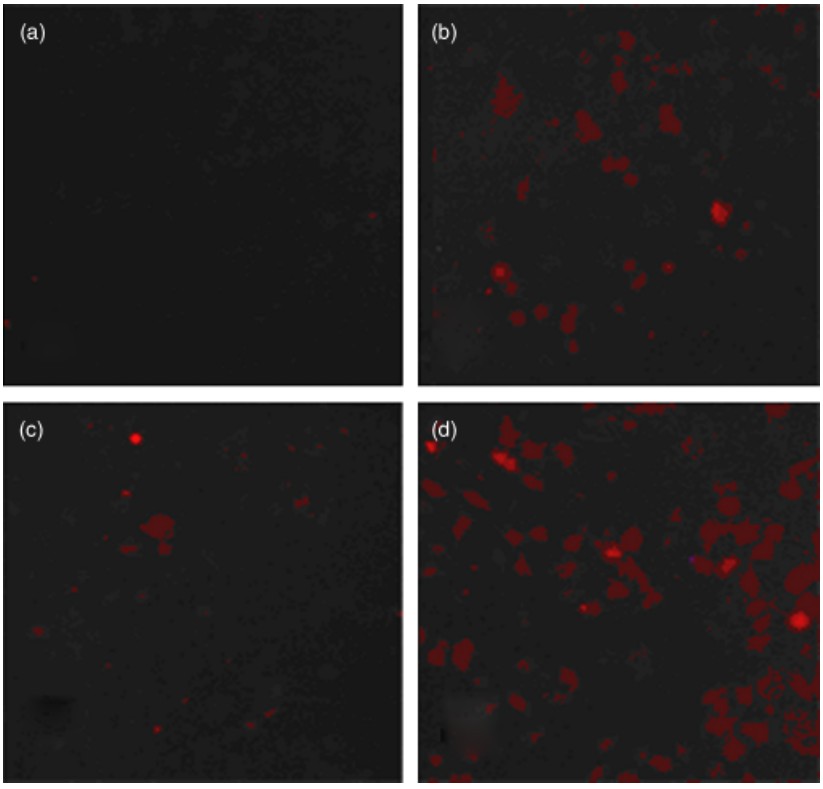

Fig. 6. Caspase-9 activation in HeLa cells exposed to: medium alone (a), actinomycin D for 4 h (b), lethal factor for 2 h (c) and lethal factor for 4 h (d).

cleave target proteins after an aspartate residue (Alnemri *et al.*, 1996). Caspase-activated DNAse (CAD) enters the nucleus and degrades chromosomal DNA (Enari *et al.*, 1998; Sakahira *et al.*, 1998).

Cleavage of the actin cytoskeleton by lethal factor was evident even after 1 h and thus resembled that caused by *C. difficile* toxins A and B. Both of these toxins are internalized by the cells and although their action differs in many details, the actin cytoskeleton seems to be their major target (Ciesielski-Treska *et al.*, 1989; Fiorentini *et al.*, 1989). Taking into account the similarity of action of both *C. difficile* toxins and lethal factor on the actin cytoskeleton it is conceivable that the latter agent also acts after internalization.

Exposure to lethal factor stimulated caspases in HeLa cells, as evidenced by the disruption of the actin cytoskeleton and increased numbers of caspase-9 and pan-caspase-positive cells. These observations, similar to those of Mashima *et al.*(1999) made in another system, suggest that cell death caused by lethal factor is caspase activation-dependent (Abraham & Shaham, 2004). The TUNEL assay demonstrated fragmentation of DNA typical for the late degradation phase of the apoptotic cells (Kroemer *et al.*, 1995). Similarly, chromatin condensation and nuclear fragmentation leading to formation of micronuclei and apoptotic bodies observed in HeLa cells is in agreement with classical descriptions of apoptosis at the ultrastructural level (Kerr *et al.*, 1972).

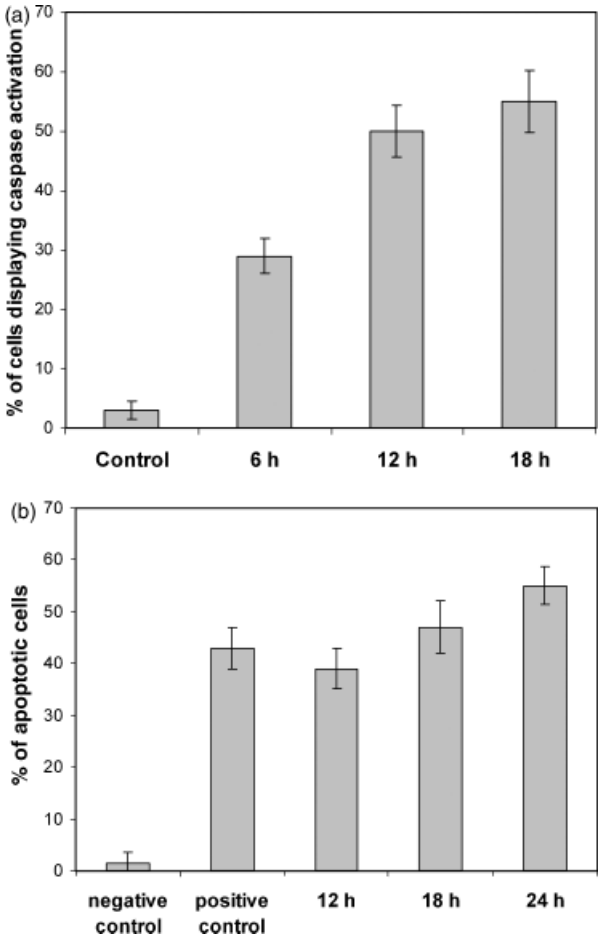

Fig. 7. Demonstration of apoptotic activity of *Clostridium histolyticum* lethal factor. (a) Activation of pan-caspase in HeLa cells exposed to 1 U of lethal factor. In each group 400 cells were analysed. Cells cultured in medium alone served as the control. (b) DNA fragmentation assay (TUNEL). HeLa cells were exposed to 1 U of lethal factor for 12, 18 and 24 h. Cells cultured in the medium alone served as the negative control and mouse thymocytes exposed to anti-FAS antibody, as supplied by the manufacturer, as the positive control. Error bars represent SD.

Ultrastructural observations showed also dilatations of the smooth endoplasmic reticulum leading to vacuolation of the cytoplasm. Such changes evoked by apoptotic agents have already been observed (Porter *et al.*, 1971; Cosulich *et al.*, 1997; Lowe *et al.*, 2004) and represent a well-known phenomenon. Given that *C. histolyticum* produces not only lethal factor but also vacuolating cytotoxin it is important to distinguish between effects caused by both these agents. Exposure to vacuolating toxin caused marked vacuolation of cytoplasm with numerous vacuoles assuming much larger dimensions than those observed in cells exposed to lethal factor. Furthermore, it stimulated an increase of neutral red accumulation, an effect not observed in cells treated with lethal factor. Finally, cells exposed to lethal factor displayed nuclear changes typical for apoptosis whereas vacuolating cytotoxin killed cells by necrosis (Jankowska-Steifer *et al.*, 2006). Thus, the effects of the action of both *C. histolyticum* toxins can be easily distinguished by biochemical and morphological criteria. The striking feature of the pathological changes produced in animals by the culture medium of *C. histolyticum* was the predominance of pycnosis and karyorexis (traditional names usually corresponding to apoptosis) in nuclei of various organs (Pasternack & Bengtson, 1940). These observations are in good agreement with changes observed in this study of HeLa cells exposed to *C. histolyticum* lethal factor and can be helpful in explaining the pathological mechanism of serious infections caused by this microorganism.

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